MTSEA prevents ligand binding to the human melanocortin-4 receptor by modification of cysteine 130 in transmembrane helix 3

Alan Cox[a,*], Dan Donnelly[b], Manminder Kaur[b,1], Sharon C. Cheetham[c,2], Victor B. Cockcroft[c], John B.C. Findlay[a]

[a] *School of Biochemistry and Microbiology, University of Leeds, Leeds LS2 9JT, UK*
[b] *School of Biomedical Sciences, University of Leeds, Leeds LS2 9JT, UK*
[c] *Knoll Pharmaceuticals, Pennyfoot Street, Nottingham NG1 1GF, UK*

Received 30 July 2004; revised 7 October 2004; accepted 18 November 2004

Available online 8 December 2004

Edited by Irmgard Sinning

Abstract **We have investigated the effect of the sulfhydryl-reactive reagent, methyl thiosulfonate ethylammonium (MTSEA), on ligand binding to the human melanocortin-4 (MC4) receptor stably expressed in HEK-293 cells. MTSEA inhibited binding of the agonist, ^{125}I-NDPα-MSH, and the antagonist, ^{125}I-SHU9119, in a concentration-dependent manner. Pre-incubation of cells with either the agonist or antagonist protected from subsequent MTSEA inhibition of radioligand binding. Mutation of Cys130 in transmembrane helix 3 to alanine, whilst not affecting ligand binding, led to a complete loss of the inhibitory effect of MTSEA. Since other types of sulfhydryl-reactive reagents had no effect on ligand binding, we conclude that covalent modification of Cys130 by MTSEA disrupts ligand binding by neutralising a close-by negative charge, most likely on Asp126.**

Keywords: Melanocortin-4 receptor; MTSEA; α-MSH; G protein-coupled receptor

1. Introduction

The melanocortins (adrenocorticotropic hormone, α-, β-γ-melanocyte stimulating hormone [MSH]) are short biologically active peptides, of related sequence, derived from tissue-specific cleavage of a precursor protein, proopiomelanocortin and all contain the conserved core sequence, -His-Phe-Arg-Trp-, which is crucial for their biological activity. Their biological effects are mediated by a group of membrane receptors (the melanocortin (MC) receptors) which belong to the G protein-coupled receptor (GPCR) super-family, the single most abundant family of membrane receptor proteins encoded by the eukaryotic genome. GPCRs are characterised by the presence of seven membrane spanning α-helices and are subdivided into several families according to function and amino acid sequence similarity. The MC receptors belong to family A, the rhodopsin-like family, and to date five MC receptors (MC1–MC5) have been characterised and cloned. They share a high degree of sequence identity and stimulate the cyclic AMP second messenger signal transduction pathway. However, the MC receptor subtypes are localised in different tissues and have specific physiological roles. These include regulation of pigmentation (MC1 receptor), glucocorticoneogenesis (MC2 receptor), energy homeostasis and appetite (MC3 and MC4 receptors) and exocrine gland secretion (MC5 receptor) [1–3].

There is a considerable body of evidence for MC4 receptor involvement in the control of food intake and energy balance both in rodents and in humans. MC4 receptors are especially abundant in the hypothalamus, the major centre within the brain involved in the control of energy homeostasis [4]. MC4 receptor knockout mice display hyperphagia and severe obesity [5] and transgenic mice that overexpress AgRP (an endogenous antagonist of MC1 and MC4 receptors) display an obese phenotype [6]. In rats, administration of the MC4 receptor agonist, MTII, reduces food intake and body weight [7], whereas administration of the highly selective MC4 receptor antagonist, HS014, induces sustained feeding and obesity [8]. In humans, administration of MC4 receptor agonists decreased body fat levels [9] and several native point mutations in the MC4 receptor have been linked to obesity [10]. Consequently, the MC4 receptor represents a major potential pharmacological target for the treatment of obesity.

Using the classical mutagenic approach, several recent publications have highlighted putative ligand-binding residues in the MC receptors. Briefly, two crucial types of interactions have been proposed: firstly, an ionic interaction between the positively charged Arg^{8} of NDPα-MSH ([Nle^{4}, D-Phe^{7}]α-MSH) and one or more acidic residues on the receptor; and secondly, a putative hydrophobic cluster involving the side-chains of Phe^{7} and Trp^{9} in the ligand and hydrophobic side-chains on transmembrane helices (TMs) 4, 5 and 6 in the receptor [11,12]. In human MC4 receptors, alanine substitution of Asp122 or Asp126 in TM3 resulted in decreases in affinity for NDPα-MSH of 5- and 1000-fold, respectively [11]. On the basis of the similar affinities of WT- and Asp122Ala

*Corresponding author. Fax: +44 113 3433167.
E-mail address: a.cox@leeds.ac.uk (A. Cox).

[1] Present address: National Heart and Lung Institute, Thoracic Medicine, Guy Scadding Building, Dovehouse Street, London SW3 6LY, UK.
[2] Present address: RenaSci Consultancy Ltd., BioCity, Nottingham NG1 1GF, UK.

Abbreviations: GPCR, G protein-coupled receptor; IAA, iodoacetamide; MC, melanocortin; α-MSH, α-melanocyte-stimulating hormone; MTS, methane thiosulfonate; MTSEA, MTS ethylammonium; NEM, *N*-ethyl maleimide; NDPα-MSH, [Nle^{4}, D-Phe^{7}]α-MSH; SHU9119, acetyl-Nle^{4}-cyclo[Asp^{5}, D-2-Nal^{7}, Lys^{10}]α-MSH(4-10) amide; TM, transmembrane

0014-5793/$22.00
doi:10.1016/j.febslet.2004.11.087

mutant receptors for Nle[8]-substituted NDPα-MSH, a direct molecular interaction between Arg[8] of the ligand and Asp122 has been proposed [11]. However, since NDPα-MSH was observed to have a greater binding affinity than Nle[8]-NDPα-MSH at the Asp122Ala mutant receptor, it seems likely that the ligand Arg[8] residue interacts with one or more receptor residues in addition to Asp122. Potentially, these could include any of five other acidic residues within the TM helices which, when substituted, also reduce ligand binding.

The use of highly specific cysteine-modifying reagents has proved an important tool in studies of structure/function relationships in a number of membrane proteins including ion channels, transporters and receptors [13–18]. An important approach is the substituted-cysteine-accessibility method in which introduced cysteines are tested for their susceptibility to modification by water-soluble methanethiosulfonate (MTS) reagents to determine buried, exposed and/or functionally important positions. The most commonly used water-soluble MTS reagent is the positively charged analogue, MTS ethylammonium (MTSEA), which converts the neutral cysteine side-chain to a positively charged group resembling a lysine side-chain. This reagent has been used to demonstrate the presence of a water-accessible cysteine within the binding-site crevice of the human dopamine D2 receptor [19] and also demonstrated the rotation of TM6 (upon receptor activation) by the appearance of an MTSEA-accessible cysteine in a constitutively active mutant of the β_2-adrenergic receptor not accessible in the wild-type receptor [20]. The effect of MTSEA on ligand binding, in conjunction with site-directed mutagenesis, has also been used to define intramolecular contacts in the neurokinin-2 tachykinin receptor [21]. Here, we describe experiments on the effect of MTS reagents on ligand binding to the human MC4 receptor and identify particularly sensitive positions.

2. Materials and methods

2.1. Materials

The expression vector, pcDNA3MC4-R, containing cDNA for the human MC4 receptor was provided by Knoll Pharmaceuticals (Nottingham). ^{125}I-NDPα-MSH and ^{125}I-acetyl-Nle[4]-cyclo[Asp[5], D-2-Nal[7], Lys[10]]α-MSH(4-10) amide (SHU9119) were obtained from Perkin Elmer Life Sciences (Boston, MA) and MTS reagents from Toronto Research Chemicals Inc. (Ontario, Canada). Non-radioactive peptides were purchased from Bachem (St. Helens, UK), cell culture reagents from Invitrogen (Paisley, UK) and protease inhibitor cocktail tablets from Roche (Lewes, UK). Oligonucleotide primers were synthesised by Sigma-Genosys Ltd. (Pampisford, UK).

2.2. Mutagenesis

Point mutations were introduced into the MC4 receptor sequence using the QuikChange® Site-Directed Mutagenesis Kit (Stratagene, Amsterdam, The Netherlands) and the pcDNA3MC4-R vector as template. Correct DNA sequences were confirmed by ABI PRISM™ Dye Terminator Cycle Sequencing (Perkin–Elmer).

2.3. Receptor expression in HEK-293 cells

Human embryonic kidney cells (HEK-293) were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% foetal calf serum, 100 U/ml penicillin, 100 μg/ml streptomycin and Glutamax™ (Invitrogen). Cells were transfected with pcDNA3 vectors containing the wild type and mutant MC4 receptor sequences using SuperFect® Transfection Reagent (Qiagen, Crawley, UK) and stable clones selected with the antibiotic, geneticin sulfate (G418), essentially as previously described [22]. In all cases, several clones of each mutant were tested and the highest binding clones selected for further analysis.

2.4. Radioligand binding assays

Radioligand binding was carried out using intact HEK-293 cells suspended in a binding buffer comprising serum-free DMEM supplemented with 0.2% (w/v) bovine serum albumin and one protease inhibitor cocktail tablet per 50 ml. Typically, ^{125}I-SHU9119 binding was carried out in a final volume of 0.2 ml binding buffer containing 30 pM ^{125}I-SHU9119 and 2×10^5 cells for 45 min at 22 °C. Non-specific binding was defined as the level of binding detected in the presence of 1 μM unlabelled SHU9119. Cell-bound radioactivity was recovered by rapid vacuum filtration through glass-fibre paper (Whatman GF/B filters pre-soaked in 1% w/v polyethyleneimine) using a Brandel cell harvester and unbound ligand removed by three washes with 3 ml ice-cold PBS. Filter-bound radioactivity was measured in a gamma counter (RiaStar 5405 counter; Packard, Pangbourne, UK). ^{125}I-NDPα-MSH binding was carried out in a similar manner except that the final assay volume was 0.1 ml and ^{125}I-NDPα-MSH was used at 140 pM. Non-specific binding was defined as the level of binding detected in the presence of 1 μM unlabelled NDPα-MSH.

2.5. MTSEA treatment of cells

Cells were treated with MTSEA for 10 min at 22 °C in 0.25 ml binding buffer and then washed by addition of 1 ml binding buffer followed by centrifugation in a microfuge ($5000 \times g$, 2 min) and resuspension of the cell pellets in fresh binding buffer.

2.6. cAMP accumulation assays

cAMP accumulation in HEK-293 cells was determined by the method of Salomon et al. [22] as reported previously. Briefly, [^{3}H]adenine pre-loaded cells on 6-well plates were stimulated with various concentrations of agonist for 12 min at 37 °C and reactions terminated by addition of ice-cold 5% (w/v) TCA containing an internal column standard of [^{14}C]cAMP. Cyclic AMP was then isolated from the TCA extracts by sequential column chromatography on Dowex 50W-X8 and alumina resins. ^{3}H and ^{14}C dpm were determined by dual isotope liquid scintillation counting and the cell-derived [^{3}H]cAMP dpm adjusted for the recovery of the [^{14}C]cAMP internal standard from individual columns.

2.7. Data analysis

All measurements (except where stated) were made in triplicate in three independent experiments and values quoted and depicted graphically are the means of the independent determinations with the SEM. In competition binding experiments, counts were normalised to the maximal specific binding within each data set and the IC_{50} values calculated with a single site-binding model fitted using a non-linear regression analysis with the aid of GraphPad PRISM 3.0 software (San Diego, CA). In a similar fashion, EC_{50} values were calculated from the fit of sigmoidal concentration–response curves. pIC_{50} refers to $-\log IC_{50}$ and pEC_{50} refers to $-\log EC_{50}$. The significance of differences between values was determined by the comparison of mean values using a two tailed unpaired Student's t test.

2.8. Molecular modelling

The three-dimensional structure of rhodopsin [25] was used as a template on which a model of the 7 TM helices of the MC4 receptor was built by replacing equivalent side-chains using SYBYL v6.3. The model was energy minimised using 2000 steps of conjugated gradient optimisation with the Tripos forcefield.

3. Results

MTSEA, applied extracellularly to cells expressing wild-type MC4 receptors, inhibited binding of both the agonist, ^{125}I-NDPα-MSH and the antagonist, ^{125}I-SHU9119 in a similar concentration-dependent manner (Figs. 1A and B). The binding of both ligands was particularly altered upon increasing the

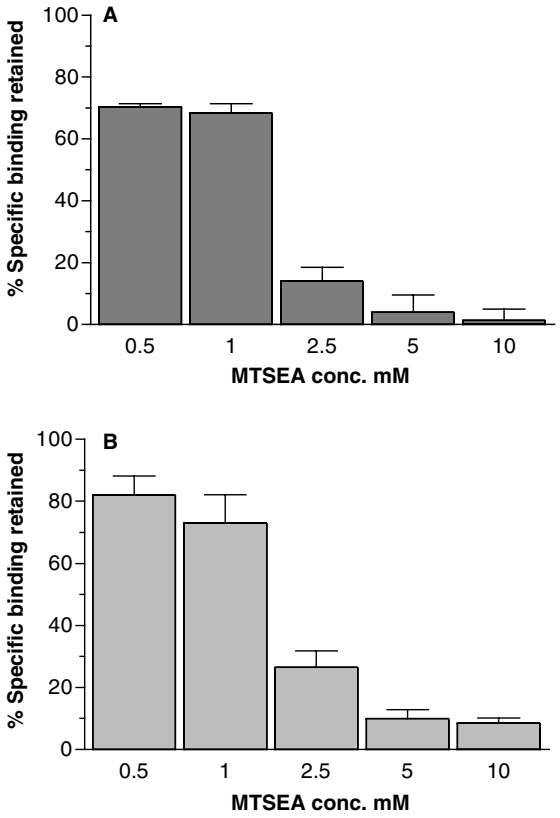

Fig. 1. The effect of MTSEA on ^{125}I-NDPα-MSH and ^{125}I-SHU9119 binding to wild type MC4 receptors stably expressed in HEK-293 cells. Cells were treated with the indicated concentrations of MTSEA for 10 min, washed and then assayed for, (A) ^{125}I-NDPα-MSH and (B) ^{125}I-SHU9119 binding. Percent specific binding retained refers to the level of specific binding after MTSEA treatment normalised to the level of specific binding in untreated cells. 100% specific binding is defined as total binding minus non-specific binding (binding in the presence of 1 μM unlabelled peptide) in untreated cells.

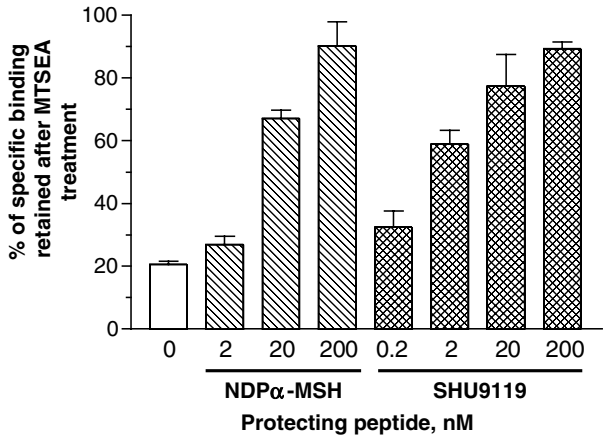

Fig. 2. Protection of MTSEA inhibition of ^{125}I-SHU9119 binding to the MC4 receptor by co-incubation with peptide ligands. Cells stably expressing wild type MC4 receptors were incubated with the indicated concentrations of either NDPα-MSH or SHU9119 for 30 min at 22 °C. Each sample was then divided into two aliquots, to which binding buffer was added to one and MTSEA added to a final concentration of 2.5 mM to the other and the incubation continued for a further 10 min. Cells were pelleted in a microfuge and resuspended in 1 ml of isotonic glycine buffer, pH 3.0 (25 mM glycine, 137 mM NaCl, 2 mM $CaCl_2$, 1 mM $MgSO_4$ and 0.2% (w/v) BSA, pH 3.0), and incubated for 20 min at 22 °C. This treatment had previously been found to efficiently remove radioligands from the MC4 receptor. Cells were then spun down again and washed further in 0.5 ml binding buffer and then assayed for ^{125}I-SHU9119 binding. For each concentration of protecting peptide, 100% specific binding is defined as the level of binding observed in samples not treated with MTSEA.

MTSEA concentration from 1 to 2.5 mM with 50% inhibition occurring between these concentrations. At the highest concentration, 10 mM MTSEA produced an almost complete block of both agonist and antagonist binding. The inhibitory effect of MTSEA on ^{125}I-SHU9119 binding was reduced by separate co-incubation with either NDPα-MSH or SHU9119 in a concentration-dependent manner (Fig. 2). At a concentration of 200 nM, both ligands achieved almost complete protection from the action of MTSEA. SHU9119 protected more effectively than NDPα-MSH, consistent with their relative affinities for the MC4 receptor [23,24].

The above observations indicate the presence of a water-accessible cysteine residue(s) within or close to the peptide ligand-binding site. The MC4 receptor contains 15 cysteines but when a model of the MC4 receptor sequence was built based on the structure of bovine rhodopsin [25], seven of these occupy positions that are unlikely to be accessible to water-soluble reagents added extracellularly (Fig. 3). Cys84(TM2), Cys138(TM3) and Cys172(TM4) are considered to be too close to the interior of the protein, Cys293(TM7) is orientated towards the membrane lipid and Cys318, Cys319 and Cys326 are located within the intracellular C-terminal domain. In order to identify the MTSEA-susceptible cysteine, we mutated the remaining eight cysteine residues individually to alanine. These were Cys40 (N-terminal domain), Cys130 (TM3), Cys177 (TM4), Cys196 (TM5) and Cys257 (TM6) and three within the third extracellular loop, Cys271, Cys277 and Cys279 (Fig. 3). When expressed in HEK-293 cells, these eight individual Cys/Ala MC4 receptor mutants were found to have no effect on either antagonist binding or receptor activation as assessed by ^{125}I-SHU9119 binding and NDPα-MSH induced cAMP accumulation, respectively (Table 1). The effect of MTSEA on ^{125}I-SHU9119 binding to these mutants was also tested (Fig. 4). At both 1 and 2.5 mM MTSEA, binding to the Cys130Ala mutant was unaffected, whereas the other seven mutants behaved similarly to wild-type MC4 receptors in that specific binding was reduced to 50–75% for 1 mM and 20–50% for 2.5 mM of the binding observed in untreated samples.

The effect of other cysteine-reactive reagents on ^{125}I-SHU9119 binding to wild-type MC4 receptors was also examined (Fig. 5A). At a concentration of 10 mM, MTSPA (positively charged), MTSES (negatively charged), *N*-ethyl maleimide (NEM) and iodoacetamide (IAA) essentially had no effect on ligand binding, whereas in the same experiments 10 mM MTSEA inhibited binding by more than 95%. To determine whether or not these reagents reacted with the MTSEA-sensitive cysteine, treated cells were subjected to an additional treatment with MTSEA. In all cases, the additional MTSEA treatment resulted in a loss of more than 90% of specific binding, indicating that MTSPA, MTSES, NEM and IAA do not react with the MTSEA-sensitive cysteine.

4. Discussion

Our data show that mutation of eight cysteine residues individually to alanine in the human MC4 receptor had no

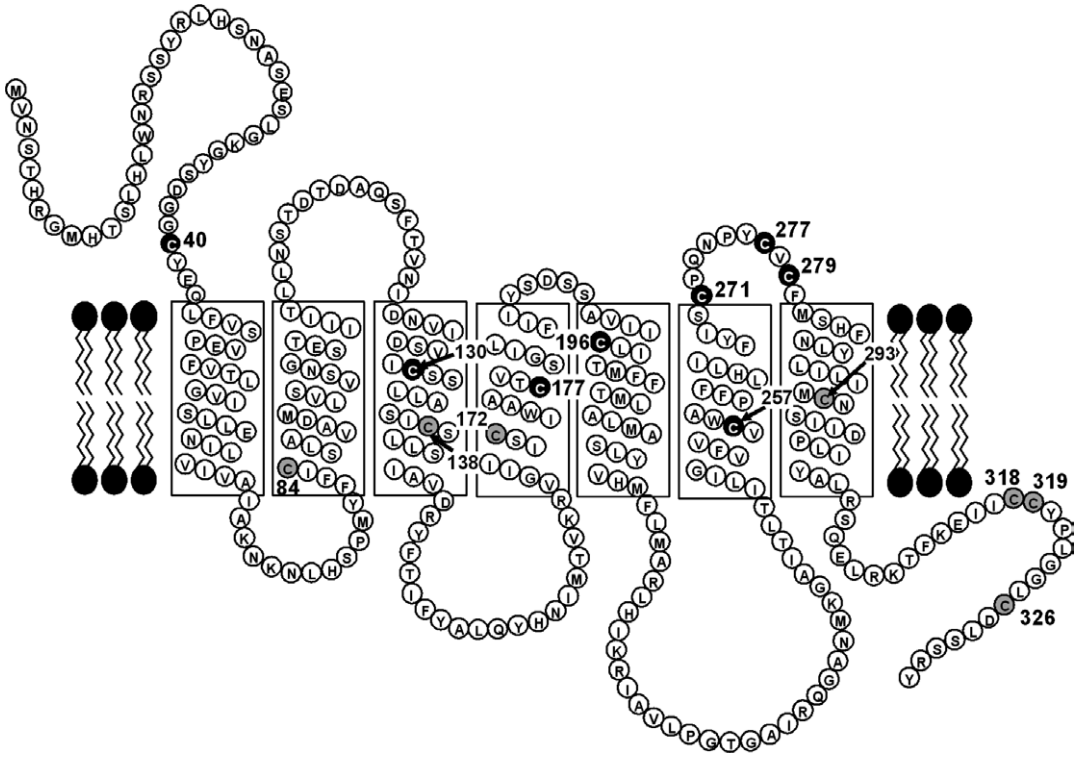

Fig. 3. Two-dimensional snake representation of the human MC4 receptor showing the approximate extent of the seven transmembrane helices and the position of all 15 cysteine residues. The eight cysteine residues that were individually mutated to alanine in this study are shown as white letters on a black background. The remaining seven cysteine residues are shown as black letters on a grey background. Further details are given in the text.

significant effect on ligand binding. This suggests that none of these residues make a crucial contribution to the binding of ligands. Some of these cysteine residues have been mutated by others and also found to have no effect on binding. These include Cys130 and 196 in the human MC4 receptor [11], Cys122 in the mouse MC4 receptor (equivalent to Cys130 in human MC4 receptor) [12] and in the mouse MC1 receptor, mutation of cysteines equivalent to the MC4 receptor cysteines 40, 130, 196 and 277 [26]. Mutation of the conserved cysteine in the N-terminal domain of the MC1 receptor from different species (Cys40 in human MC4 receptor) resulted in different outcomes and hence its role remains to be clarified [26,27].

Table 1
Antagonist binding and agonist activation properties of wild type and mutant MC4 receptors

Receptor mutation	SHU9119 binding[a] pIC_{50}	NDPα-MSH activation[b] pEC_{50}
WT	9.20 ± 0.06 (0.63)	7.70 ± 0.24 (20.0)
C40A	9.00 ± 0.07 (1.00)	7.89 ± 0.24 (12.8)
C130A	9.05 ± 0.03 (0.89)	7.38 ± 0.03 (41.7)
C177A	9.18 ± 0.07 (0.66)	7.55 ± 0.12 (28.2)
C196A	9.37 ± 0.06 (0.43)	7.76 ± 0.11 (17.4)
C257A	9.16 ± 0.19 (0.69)	7.64 ± 0.11 (22.9)
C271A	9.41 ± 0.22 (0.39)	7.37 ± 0.15 (42.7)
C277A	9.31 ± 0.06 (0.49)	7.45 ± 0.11 (35.5)
C279A	9.39 ± 0.12 (0.41)	7.73 ± 0.07 (18.6)

Competition binding assays and agonist concentration response curves were performed as described in the methods section. (a) For SHU9119 binding, $n = 3$ except for C271A ($n = 2$), C277A ($n = 4$) and C279A ($n = 4$). Data are expressed as pIC_{50} ± SEM followed by, in brackets, IC_{50} values in nM. (b) For NDPα-MSH activation, $n = 3$ except for C130A ($n = 2$), C277A ($n = 4$) and C279A ($n = 2$). Potency values are expressed as pEC_{50} ± SEM followed by, in brackets, EC_{50} values in nM. Values for all mutants for antagonist binding and receptor activation were not significantly different from the values for the wild type MC4 receptor ($P > 0.1$).

It is clear that the three cysteines in extracellular loop 3 (cysteines 271, 277 and 279 in the human MC4 receptor) are conserved amongst all MC receptor subtypes and in the MC1 receptor the outer two of these are postulated to form a disulfide bond, which is crucial for receptor function [26]. It is surprising, therefore, that here, for the MC4 receptor we observed no effect of substituting any of the cysteines in the third extracellular loop. This may indicate the lack of a similar disulfide bond in the MC4 receptor or that, if present, such a bond is not important for ligand binding. Clearly, there are important differences in the effects of cysteine residue substitution between different MC receptor subtypes and also species differences for similar subtypes which have yet to be fully understood.

We have demonstrated that the positively charged cysteine-modifying reagent, MTSEA, blocks ligand binding in the human MC4 receptor by a specific reaction with Cys130 found close to the middle of TM3. Binding of the agonist, NDPα-MSH and the antagonist, SHU9119 was similarly inhibited by MTSEA in a concentration-dependent manner. Of the fifteen cysteine residues present in the MC4 receptor, only eight occupy positions that could potentially be involved in ligand binding. When each of these was individually mutated to alanine, seven retained sensitivity to MTSEA, whereas the Cys130Ala mutant receptor was no longer affected by MTSEA (Fig. 4). This is similar to observations made for the human dopamine D2 receptor in which Cys118, also in TM3, was modified by MTSEA causing inhibition of antagonist binding

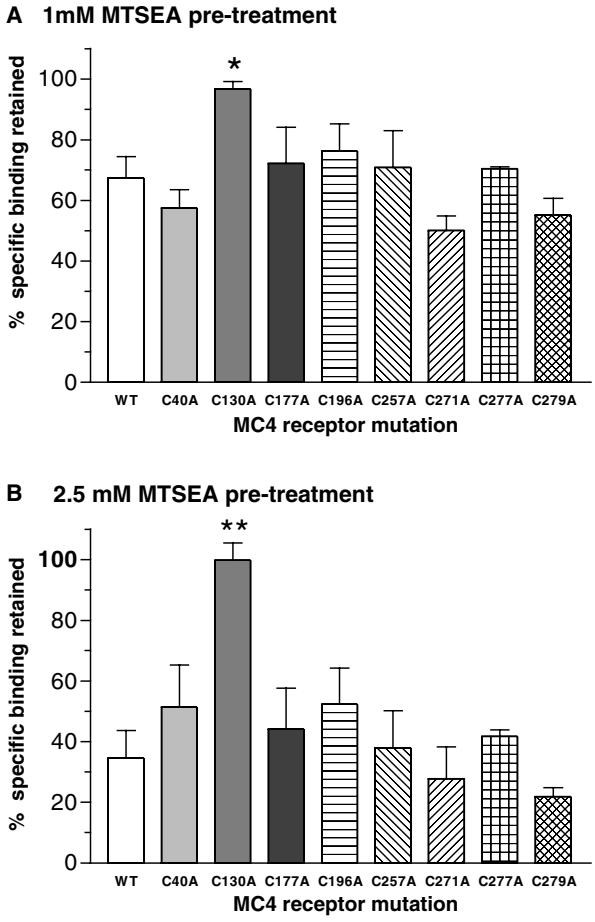

Fig. 4. Effect of MTSEA pre-treatment on ^{125}I-SHU9119 binding to wild type and mutant MC4 receptors. Cells expressing wild type and mutant MC4 receptors were treated with either 1 or 2.5 mM MTSEA for 10 min at 22 °C, washed and then assayed for specific ^{125}I-SHU9119 binding. The values for the C130A mutant were significantly different from the wild type value (for 1mM MTSEA, $*P < 0.025$ and for 2.5 mM MTSEA, $**P < 0.002$).

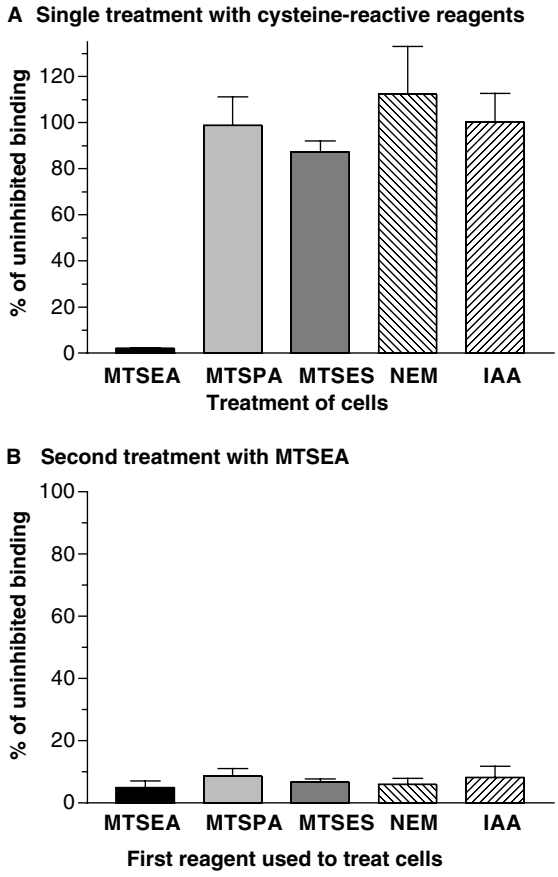

Fig. 5. Effect of various SH-reagents on ^{125}I-SHU9119 binding to wild type MC4 receptors. (A) Cells expressing wild type MC4 receptors were treated with the SH-reagents shown (all at 10 mM) for 10 min at 22 °C, washed and then assayed for specific ^{125}I-SHU9119 binding. The level of binding obtained was related to the level of binding in untreated cells (100% binding). (B) Cells expressing wild type MC4 receptors were treated with the SH-reagents shown for 10 min at 22 °C, washed and then all samples treated for a further 10 min with 10 mM MTSEA. After a further washing step, cells were assayed for ^{125}I-SHU9119 binding. The level of binding obtained was related to the level of binding in untreated cells (100% binding).

[19]. However, alignment of the MC4 and D2 receptor sequences demonstrates that the MTSEA-sensitive cysteines are located in different positions, Cys118 in the D2 receptor being one α-helical turn deeper into the lipid bilayer than Cys130 in the MC4 receptor.

Mutation of Cys130 to alanine had no significant effect on agonist or antagonist binding (Table 1), suggesting that there is no direct interaction between Cys130 and either ligand and is not itself critical for ligand binding. However, since pre-incubation with agonist or antagonist reduced the inhibitory effect of MTSEA (Fig. 2), this suggests that Cys130 is close to the peptide binding-site. The lack of effect of IAA and NEM on ligand binding and, indeed, the lack of reactivity with Cys130 (Fig. 5) suggests a degree of specificity in the reaction of MTSEA with Cys130 or that this residue is not readily accessible to all reagents.

To further examine the nature of this specificity, we tested the effects of other MTS reagents on ligand binding. We found that the positively charged analogue, MTSPA, which is just one carbon atom longer than MTSEA, had no effect on ligand binding and that it did not react with Cys130 (Fig. 5). The negatively charged analogue, MTSES, similarly had no effect on ligand binding and did not react with Cys130. Also, as indicated earlier, the uncharged, more conventional, cysteine-reactive reagents, NEM and IAA, behaved similarly.

We speculate that the unique effect of MTSEA on the MC4 receptor resides in an ionic interaction of the positively charged amine group on MTSEA and the negatively charged acidic residues, Asp126 and Asp122, respectively, one and two α-helical turns away from Cys130. These three residues are stacked upon each other down one face of TM3 that lines the ligand-binding crevice (Fig. 6). This is similar to suggestions made for the D2 receptor in which the positive charge on MTSEA is thought to bind to an aspartate residue one helical turn away from the modified cysteine in TM3 [19]. The aspartic acid residues Asp126 and Asp122 in the MC4 receptor are thought to make a crucial ionic interaction with the positively charged arginine side-chain in core position 8 of α-MSH based ligands [11]. Thus, the inhibitory action of MTSEA at the MC4 receptor may be explained by its positive charge occupying a position crucial to ligand binding, a position normally occupied by a positive charge on peptide ligands. In our modelling of the TM regions of the MC4 receptor, in

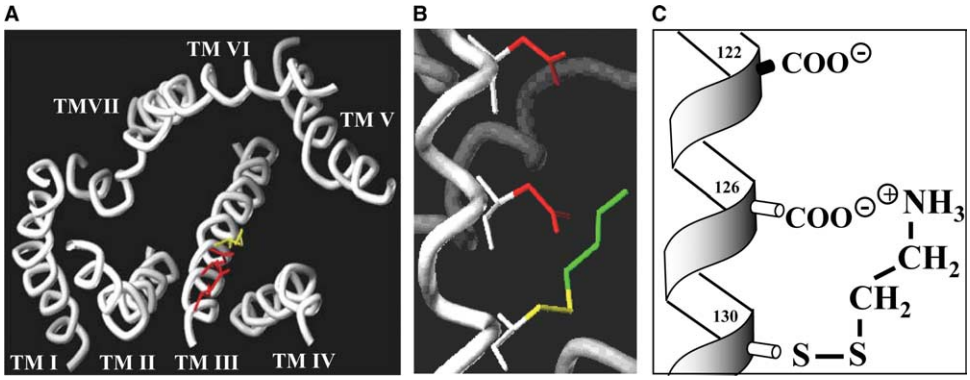

Fig. 6. Molecular model of the transmembrane helices of the human MC4 receptor depicting the amino acid residues in TM3 involved in interaction with MTSEA. The model was built as described in Section 2 using the three-dimensional structure of rhodopsin as a template. (A) Molecular model of the human MC4 receptor as viewed from an extracellular position perpendicular to the plane of the plasma membrane. The model was built upon the known structure of bovine rhodopsin [25]. The α-helical backbone structure of the seven transmembrane helices is shown in various shades of grey and the side-chains of Cys130 (yellow), Asp126 (red) and Asp122 (red) in TM3 are highlighted. (B) A close-up of part of the model depicting the most extracellular region of TM3. Here, the view is side-on and shows the ''stacked'' nature of the side-chains of Cys130, Asp126 and Asp122 down one face of the helix. Cys130 is shown following modification by MTSEA (coloured green). (C) A schematic representation of the model shown in (B).

which we include the MTSEA modification of Cys130 (Fig. 6), it is clear that the positive charge on the modified side-chain can only reach as far as the side-chain of Asp126 and thus unlikely to interact with Asp122. Thus, neutralising only the charge on Asp126 is sufficient to block ligand binding.

For the positively charged MTSPA, which does not bind to Cys130, it may be that, being one carbon atom longer, it is too bulky to bind in this region. Alternatively, the reactive methane thiosulphonate group of MTSPA may not be correctly orientated for the reaction with the side-chain of Cys130. MTSES had no effect on ligand binding since, being negatively charged, it is likely to be excluded from the negatively charged region of TM3. NEM and IAA, both being uncharged and relatively bulky, are likely to experience no electrostatic attraction to the charged region of TM3 and steric hindrance may also prevent access to the side-chain of Cys130. Thus, treatment with these cysteine-reactive reagents leaves Cys130 untouched and therefore free to react upon a further treatment with MTSEA (Fig. 5).

In summary, we have demonstrated for the first time that treatment of the human MC4 receptor with the cysteine-modifying reagent, MTSEA, prevents the binding of agonist and antagonist ligands and identified the site of modification as Cys130 in TM3. This indicates the proximity of Cys130 to the ligand binding-site. Although the residue itself is not crucial for ligand binding, nevertheless, there may well be a cavity that allows correct docking of the ligand in this region. Thus, further analysis of this TM region with other probes may help in an appreciation of the size and depth of the binding pocket.

Acknowledgements: This project was supported by the Wellcome Trust and previously by Knoll Pharmaceuticals (Nottingham). We thank Mr. John Sinfield for help with Fig. 3.

References

[1] Mountjoy, K.G., Robbins, L.S., Mortrud, M.T. and Cone, R.D. (1992) The cloning of a family of genes that encode the melanocortin receptors. Science 257, 1248–1251.

[2] Chen, A.S., Marsh, D.J., Trumbauer, M.E., Frazier, E.G., Guan, X.M., Yu, H., Rosenblum, C.I., Vongs, A., Feng, Y., Cao, L.H., Metzger, J.M., Strack, A.M., Camacho, R.E., Mellin, T.N., Nunes, C.N., Min, W., Fisher, J., Gopal-Truter, S., MacIntyre, D.E., Chen, H.Y. and Van der Ploeg, L.H.T. (2000) Inactivation of the mouse melanocortin-3 receptor results in increased fat mass and reduced lean body mass. Nat. Genet. 26, 97–102.

[3] Chen, W.B., Kelly, M.A., OpitzAraya, X., Thomas, R.E., Low, M.J. and Cone, R.D. (1997) Exocrine gland dysfunction in MC5-R-deficient mice: Evidence for coordinated regulation of exocrine gland function by melanocortin peptides. Cell 91, 789–798.

[4] Mountjoy, K.G., Mortrud, M.T., Low, M.J., Simerly, R.B. and Cone, R.D. (1994) Localization of the Melanocortin-4 Receptor (Mc4-R) in Neuroendocrine and Autonomic Control-Circuits in the Brain. Mol. Endocrinol. 8, 1298–1308.

[5] Huszar, D., Lynch, C.A., FairchildHuntress, V., Dunmore, J.H., Fang, Q., Berkemeier, L.R., Gu, W., Kesterson, R.A., Boston, B.A., Cone, R.D., Smith, F.J., Campfield, L.A., Burn, P. and Lee, F. (1997) Targeted disruption of the melanocortin-4 receptor results in obesity in mice. Cell 88, 131–141.

[6] Ollmann, M.M., Wilson, B.D., Yang, Y.K., Kerns, J.A., Chen, Y.R., Gantz, I. and Barsh, G.S. (1997) Antagonism of central melanocortin receptors in vitro and in vivo by Agouti-related protein. Science 278, 135–138.

[7] Murphy, B., Nunes, C.N., Ronan, J.J., Harper, C.M., Beall, M.J., Hanaway, M., Fairhurst, A.M., Van der Ploeg, L.H.T., MacIntyre, D.E. and Mellin, T.N. (1998) Melanocortin mediated inhibition of feeding behavior in rats. Neuropeptides 32, 491–497.

[8] Kask, A., Pahkla, R., Irs, A., Rago, L., Wikberg, J.E.S. and Schioth, H.B. (1999) Long-term administration of MC4 receptor antagonist HS014 causes hyperphagia and obesity in rats. Neuroreport 10, 707–711.

[9] Fehm, H.L., Smolnik, R., Kern, W., McGregor, G.P., Bickel, U. and Born, J. (2001) The melanocortin melanocyte-stimulating hormone/adrenocorticotropin(4–10) decreases body fat in humans. J. Clin. Endocrinol. Metab. 86, 1144–1148.

[10] Hinney, A., Schmidt, A., Nottebom, K., Heibult, O., Becker, I., Ziegler, A., Gerber, G., Sina, M., Gorg, T., Mayer, H., Siegfried, W., Fichter, M., Remschmidt, H. and Hebebrand, J. (1999) Several mutations in the melanocortin-4 receptor gene including a nonsense and a frameshift mutation associated with dominantly inherited obesity in humans. J. Clin. Endocrinol. Metab. 84, 1483–1486.

[11] Yang, Y., Fong, T.M., Dickinson, C.J., Mao, C., Li, J.Y., Tota, M.R., Mosley, R., Van der Ploeg, L.H.T. and Gantz, I. (2000) Molecular determinants of ligand binding to the human melanocortin-4 receptor. Biochemistry 39, 14900–14911.

[12] Haskell-Luevano, C., Cone, R.D., Monck, E.K. and Wan, Y.P. (2001) Structure activity studies of the melanocortin-4 receptor by in vitro mutagenesis: Identification of agouti-related protein (AGRP), melanocortin agonist and synthetic peptide antagonist interaction determinants. Biochemistry 40, 6164–6179.
[13] Sun, Z.P., Akabas, M.H., Goulding, E.H., Karlin, A. and Siegelbaum, S.A. (1996) Exposure of residues in the cyclic nucleotide-gated channel pore: P region structure and function in gating. Neuron 16, 141–149.
[14] Yang, N.B., George, A.L. and Horn, R. (1996) Molecular basis of charge movement in voltage-gated sodium channels. Neuron 16, 113–122.
[15] Chen, J.G., LiuChen, S. and Rudnick, G. (1997) External cysteine residues in the serotonin transporter. Biochemistry 36, 1479–1486.
[16] Kulkarni, A.A., Haworth, I.S., Uchiyama, T. and Lee, V.H.L. (2003) Analysis of transmembrane segment 7 of the dipeptide transporter hPepT1 by cysteine-scanning mutagenesis. J. Biol. Chem. 278, 51833–51840.
[17] Javitch, J.A., Fu, D.Y., Chen, J.Y. and Karlin, A. (1995) Mapping the binding-site crevice of the dopamine D2 receptor by the substituted-cysteine accessibility method. Neuron 14, 825–831.
[18] Fu, D.Y., Ballesteros, J.A., Weinstein, H., Chen, J.Y. and Javitch, J.A. (1996) Residues in the seventh membrane-spanning segment of the dopamine D2 receptor accessible in the binding-site crevice. Biochemistry 35, 11278–11285.
[19] Javitch, J.A., Li, X., Kaback, J. and Karlin, A. (1994) A cysteine residue in the third membrane-spanning segment of the human D2 dopamine receptor is exposed in the binding-site crevice. Proc. Natl. Acad. Sci. USA 91, 10355–10359.
[20] Rasmussen, S.G., Jensen, A.D., Liapakis, G., Ghanouni, P., Javitch, J.A. and Gether, U. (1999) Mutation of a highly conserved aspartic acid in the beta2 adrenergic receptor: constitutive activation, structural instability, and conformational rearrangement of transmembrane segment 6. Mol. Pharmacol. 56, 175–184.
[21] Bhogal, N., Blaney, F.E., Ingley, P.M., Rees, J. and Findlay, J.B.C. (2004) Evidence for the proximity of the extreme N-terminus of the neurokinin-2 (NK2) tachykinin receptor to cys167 in the putative fourth transmembrane helix. Biochemistry 43, 3027–3038.
[22] Al-Sabah, S. and Donnelly, D. (2003) A model for receptor-peptide binding at the glucagon-like peptide-1 (GLP-1) receptor through the analysis of truncated ligands and receptors. Br. J. Pharmacol. 140, 339–346.
[23] Hruby, V.J., Lu, D.S., Sharma, S.D., Castrucci, A.D., Kesterson, R.A., Alobeidi, F.A., Hadley, M.E. and Cone, R.D. (1995) Cyclic Lactam Alpha-Melanotropin Analogs of Ac-Nle(4)- Cyclo Asp(5),D-Phe(7),Lys(10) Alpha-Melanocyte-Stimulating Hormone-(4–10)-Nh2 with Bulky Aromatic-Amino-Acids at Position-7 Show High Antagonist Potency and Selectivity at Specific Melanocortin Receptors. J. Med. Chem. 38, 3454–3461.
[24] Kask, A., Mutulis, F., Muceniece, R., Pahkla, R., Mutule, I., Wikberg, J.E.S., Rago, L. and Schioth, H.B. (1998) Discovery of a novel superpotent and selective melanocortin-4 receptor antagonist (HS024): Evaluation in vitro and in vivo. Endocrinology 139, 5006–5014.
[25] Palczewski, K., Kumasaka, T., Hori, T., Behnke, C.A., Motoshima, H., Fox, B.A., Le Trong, I., Teller, D.C., Okada, T., Stenkamp, R.E., Yamamoto, M. and Miyano, M. (2000) Crystal structure of rhodopsin: A G protein-coupled receptor. Science 289, 739–745.
[26] Holst, B., Elling, C.E. and Schwartz, T.W. (2002) Metal ion-mediated agonism and agonist enhancement in melanocortin MC1 and MC4 receptors. J. Biol. Chem. 277, 47662–47670.
[27] Frandberg, P.A., Doufexis, M., Kapas, S. and Chhajlani, V. (2001) Cysteine residues are involved in structure and function of melanocortin 1 receptor: Substitution of a cysteine residue in transmembrane segment two converts an agonist to antagonist. Biochem. Biophys. Res. Commun. 281, 851–857.